Detection of the A302S *Rdl* Mutation in Fipronil Bait-Selected Strains of the German Cockroach (Dictyoptera: Blattellidae)

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ABSTRACT Extensive usage and heavy reliance on insecticides have led to the development of insecticide resistance in the German cockroach, *Blattella germanica* (L.). Six field-collected strains of *B. germanica* from Singapore were used to investigate resistance to fipronil and dieldrin. The three strains (Boat Quay, Cavenagh Road, and Ghimmoh Road) with greatest resistance to fipronil were subjected to selection with fipronil bait up to the F_5 generation. Synergism assay and molecular detection of a target site mutation were used to elucidate the mechanism of fipronil resistance in these strains. With the exception of the Cavenagh Road strain, all parental strains were susceptible to dieldrin. This strain exhibited resistance to dieldrin and fipronil with resistance ratios of 4.1 and 3.0, respectively. Piperonyl butoxide and *S,S,S*-tributylphosphorotrithioate were antagonistic toward fipronil toxicity in all strains. Bait selection significantly increased fipronil and dieldrin resistance in the three chosen strains, either in topical bioassay or bait evaluations. There was a significant positive relationship [$y = (6{,}852.69 \pm 1{,}988.37)x - (708.93 \pm 1{,}226.28)$, where x = fipronil toxicity and y = dieldrin toxicity] between dieldrin and fipronil resistance levels, indicating significant cross-resistance between the insecticides. High frequencies of individuals possessing the *Rdl* gene mutation were found in the F_5 generation of the three strains selected with fipronil bait. The synergism assays indicated that monooxygenase and esterase were not involved in fipronil resistance in the strains studied herein. The A302S *Rdl* mutation was the major mechanism contributing to fipronil and dieldrin resistance in these strains.

KEY WORDS insecticide resistance, fipronil, dieldrin, cross-resistance, *Rdl* gene

The German cockroach, *Blattella germanica* (L.), is an important urban insect pest. German cockroaches are potential mechanical vectors of many pathogens, including bacteria, helminths, protozoans, and viruses (Brenner 1995; Lee 1997b, 2007; Lee and Ng 2009). Accidental ingestion or inhalation of their fecal materials and saliva may trigger allergies and asthma (Lee and Ng 2009). To manage this pest, chemical treatment remains the most effective method. However, heavy reliance on insecticide treatments and high frequency of use have led to the development of resistance to various classes of insecticide, including chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids (Lee et al. 1996, Lee 1997a, Lee and Lee 2004), phenylpyrazoles (Holbrook et al. 2003, Kristensen et al. 2005, Gondhalekar and Scharf 2012), and oxidiazines (Chai and Lee 2010). Insecticide resistance in the German cockroach has become a major problem for the pest management industry.

Fipronil, a phenylpyrazole insecticide, has been widely used against German cockroaches, especially in the form of gel bait. The mode of action of fipronil is similar to that of the cyclodienes (i.e., affecting the γ-aminobutyric acid [GABA]-gated chloride channel) (Cole et al. 1993, Hosie et al. 1995, Gant et al. 1998, Narahashi 2002, Buckingham and Sattelle 2005). Fipronil and its metabolite compounds also affect glutamate-activated chloride channels (GluCIs) (Zhao et al. 2004). Over the past 10 yr, cases of fipronil resistance in field populations of the German cockroach have been reported (Holbrook et al. 2003, Kristensen et al. 2005, Gondhalekar and Scharf 2012). Fipronil-resistant *B. germanica* appeared in the United States (Holbrook et al. 2003) and Denmark (Kristensen et al. 2005), even before the use of fipronil against cockroaches. Gondhalekar and Scharf (2012) reported fipronil resistance in GNV-R strain, a multi-insecticide-resistant field strain, of *B. germanica* after fipronil bait had been available in the United States for more than a decade, and they suggested that fipronil resistance in the German cockroach is on the rise.

Two types of mechanisms confer fipronil resistance in *B. germanica:* the P_{450} monooxygenase-mediated

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pathway and *Rdl* mutation. *Rdl* mutation is caused by conversion of alanine to serine at position 302 (using *Drosophila* notation) in the *Rdl* gene that encodes a subunit of the GABA receptor. Kristensen et al. (2005) documented *Rdl* mutation as the major mechanism conferring fipronil resistance in several Danish German cockroach populations, and Gondhalekar and Scharf (2012) reported that fipronil resistance in a field-collected *B. germanica* population was caused by combined effects of enhanced P_{450} monooxygenase and *Rdl* mutation. However, Holbrook et al. (2003) and Kristensen et al. (2005) predicted that other mechanisms could also be involved.

Recently, Chai and Lee (2010) reported broad-spectrum resistance in field populations of the German cockroach collected from Singapore. The authors discovered several strains of *B. germanica* exhibiting fipronil resistance, even though these populations had no previous or limited exposure to the insecticide. We suspect that previous exposure to dieldrin in these strains could have contributed to cross-resistance to fipronil. By using the six strains of German cockroach described by Chai and Lee (2010), we examined the relationships between fipronil and dieldrin and subsequently investigated the resistance mechanisms via a synergism study, and performed molecular detection of a target site mutation of three strains (Boat Quay, Cavenagh Road, and Ghimmoh Road) after several generations of laboratory selection by using a fipronil bait.

Materials and Methods

Insects. Six field-collected strains of German cockroach (B1 Tampines Central, Beach Road, Boat Quay, Victoria Street, Cavenagh Road, and Ghimmoh Road) were used in the topical bioassay and synergism studies (Chai and Lee 2010). Synergism studies were carried out to determine the possible mechanism of resistance in the German cockroach in vivo. A laboratory insecticide-susceptible strain, Environmental Health Institute (EHI), was used as control. All insects were reared in the laboratory and were held in polyethylene aquaria (38 by 22 by 27 cm) under environmental conditions of 26 ± 1°C, 60 ± 5% relative humidity, and a photoperiod of 12:12 (L:D) h. Dry dog food and water were provided ad libitum.

Insecticides and Synergists. Technical-grade insecticides fipronil at 95.6% purity (PESTANAL, Sigma–Aldrich Laborachemikalien GmBh, Munich, Germany) and dieldrin at 90% purity (Sigma-Aldrich Sdn. Bhd., Kuala Lumpur, Malaysia) and the synergists piperonyl butoxide (PBO) at 98% purity (FMC Corporation, Middleport, NY) and *S,S,S*-tributylphosphorotrithioate (DEF) at 98.3% purity (Miles Inc., Stilwell, KS) were used in this study. All insecticides and synergists were prepared by diluting them with technical-grade acetone (Sigma, St. Louis, MO). Goliath (0.05% fipronil) cockroach gel bait was supplied by Bentz Jaz Singapore Pte. Ltd. and Bayer Environmental Science (Singapore).

Topical Bioassay to Determine Baseline Susceptibility to Fipronil and Dieldrin With or Without Synergists. Adult males aged 1–3 wk from each of the six German cockroach strains were used in this study. For each treatment type, 10 insects were anesthetized with CO_2 (15 kPa pressure) for 5–10 s before insecticide treatment to facilitate the treatment process. One microliter of a predetermined dose of insecticide (fipronil or dieldrin) in acetone was applied on the first segment of the cockroach abdominal sternites by using a microapplicator (Burkard Scientific Ltd., Middlesex, United Kingdom) equipped with a 27-gauge needle on a 1-ml hypodermic insulin glass syringe (Fortune WG Co., Munich, Germany). The control set consisted of 10 cockroaches treated with 1.0 μl of acetone. For the fipronil synergism studies, the cockroaches were first treated with 1.0 μl of synergists PBO or DEF (or both) at doses of 100 and 30 μg per insect, respectively, on the abdominal sternites. This was done ≈2 h before treatment with 1 μl of the predetermined dose of fipronil. The control cockroaches were treated with 1.0 μl of synergist, followed by acetone.

Treated cockroaches were placed into a clean petri dish (90 mm in diameter by 15 mm in height) provisioned with dog food and a wet cotton ball. Each insecticide experiment consisted of 3–7 concentrations that resulted in >0 to <100% mortality, and the experiment was replicated 3–5 times, depending on the availability of adult males. Mortality of treated insects was recorded at 48 h posttreatment. Cockroaches were considered dead if they were unable to right themselves within 60 s after being probed with a forceps. The mean weight of an adult male for each strain was determined by weighing five groups of 10 adult males.

Bait Evaluation. The performance of fipronil cockroach gel bait was evaluated against the six cockroach strains in a choice assay. For each strain, 10 adult males were introduced into a test arena (50.0 by 37.0 by 9.0 cm). Each arena was provided with a folded cardboard harborage at the center and water in a cotton-stopped vial located under the harborage. The inner upper surface of the test arena was greased with a mixture of petroleum jelly (Vaseline, Unilever, NJ) and baby oil in a portion of 3:1 to prevent the test cockroaches from escaping. The insects were allowed to acclimatize for 1 d before introduction of bait. Dead or weak cockroaches were replaced with healthy ones immediately before the bait placement. A small dish consisting of ≈0.1 g of bait or an empty plate (as the control) was placed at one corner of the tray, and a small dish containing dry dog food was placed at the other corner of the test arena. The mortality of the test cockroaches was recorded at selected hourly intervals (normally every 1–3 h), depending on the mortality rate, until all cockroaches were dead. Dead cockroaches were removed after each count. The experimental trays were kept under the environmental conditions previously described. The experiment was replicated four times.

Bait Selection. Based on the baseline susceptibility of fipronil and dieldrin, three strains (Boat Quay,

Cavenagh Road, and Ghimmoh Road) were chosen for selection with fipronil bait to study the development of fipronil resistance after multiple generations of bait selection. For each strain, groups of German cockroach nymphs were exposed to ≈3 g fipronil cockroach gel bait without the presence of alternative food for 24–48 h to cause >80% mortality. The survivors were separated and reared in a culture tank provisioned with dry dog food and water under laboratory conditions. The selection process was repeated until the fifth generation (F_5). Adult males aged 1–3 wk of the offspring of each strain were subjected to topical dose–response and bait assays. Synergism (using PBO) was tested on the F_5 generation of the three selected strains to determine the effect of monooxygenase on fipronil resistance after selection.

Detection of the *Rdl* Mutation. Male cockroaches of the parental and F_5 generation from each fipronil-selected population were stored in 95% ethanol before DNA extraction. The cephalothorax of the cockroach was powdered in the presence of liquid nitrogen and homogenized in sodium-Tris-EDTA buffer. The DNA was extracted by conventional phenol–chloroform extraction. Genomic DNA was stored at −20°C and used as the template in polymerase chain reaction (PCR). A pair of primers designed by Hansen et al. (2005) (forward 5′-GTGCGGTCCATGGGATACTA-3′ and reverse 5′-AACGACGCGAAGACCATAAC-3′) was used in the PCR to amplify the 245-bp exon 7 (M2) region of the *Rdl* gene enclosing the A302S mutation. The 50 μl sample of PCR mix contained 50–100 ng of template DNA, forward and reverse primers (10 μM), $MgCl_2$, *Taq*DNA polymerase, and dNTPs in buffer solution. Negative controls with nuclease-free water as the template, instead of DNA, were used. All PCRs were carried out under the following temperature cycle (Gondhalekar and Scharf 2012): 94°C for 5 min (initial denaturation), 40 cycles at 94°C for 30 s (DNA denaturation), 64.3°C for 30 s (primer annealing), and 72°C for 30 s (extension), followed by a final cycle at 72°C for 10 min (final extension). Five microliters of the PCR product were verified through gel electrophoresis on an ethidium bromide-stained 1.5% agarose gel. The remaining PCR products were purified by using the LaboPass Gel PCR clean-up kit (COSMO GeneTech, Seoul, Korea) according to the manufacturer's protocol, before direct sequencing. Direct sequencing chromatograph of individuals of all cockroach strains were identified and scored into three patterns of genotypes: Ala302/Ala302, Ala302/Ser302, and Ser302/Ser302 (Fig. 1). The experiment was replicated with 20 individuals for each generation.

Statistical Analysis. Data were pooled and subjected to probit analysis by using POLO-PC (LeOra 1997). The toxicity of the insecticide was expressed as an absolute value (micrograms per gram insect) to avoid the influence of weight differences on insecticide susceptibility. The bait efficacy was presented in time (h). Significant differences in lethal dose or lethal time at 50% mortality (LD_{50} or LT_{50}) were based on the nonoverlap of the 95% fiducial limit (FL). The resistance ratio (RR_{50}) was calculated by dividing the LD_{50} or LT_{50} of a resistant strain by the corresponding value of the EHI-susceptible strain, whereas RR_{95} was calculated by using the respective values of LD_{95} or LT_{95} in susceptible and resistant strains. The RR was classified into five categories according to Lee and Lee (2004) and Chai and Lee (2010): ≤1, absence of resistance; >1 to ≤5, low resistance; >5 to ≤10, moderate resistance; >10 to ≤50, high resistance; and >50, very high resistance. The synergism ratio was determined by dividing the LD_{50} of insecticide alone with that of insecticide + synergist(s). Relationships between LD_{50} of fipronil and LD_{50} of dieldrin were evaluated by correlation analysis by using SPSS version 11.5.0 (SPSS 2002). When possible, a mathematical function was further generated by a simple linear regression model by using SPSS SigmaPlot 10.0 (SigmaPlot 2006). The ratio of enzyme level (R/S) was calculated by dividing enzyme activity or amount of resistant strains by that of an EHI-susceptible strain.

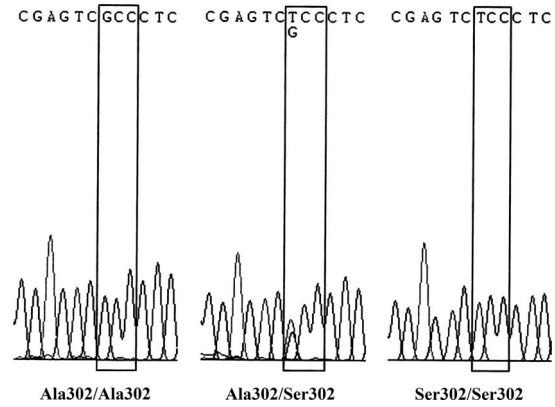

Fig. 1. Direct sequencing chromatograph of three genotypes of cockroach individuals. The 302 mutation site is boxed. Homozygous susceptible; Ala302/Ala302, heterozygous; Ala302/Ser302 and homozygous resistant; Ser302/Ser302.

Results

Fipronil and Dieldrin Susceptibility. Table 1 lists the toxicity of fipronil and dieldrin against the six strains of German cockroach. The LD_{50} for dieldrin by topical application was 4.9 μg/g. Most of the strains did not exhibit resistance to dieldrin based on LD_{50} at the 95% FL. The RR was in the range of 1.1–1.8×. Only the Cavenagh Road strain demonstrated low resistance to dieldrin, with an RR_{50} value of 4.1×, and it also exhibited low tolerance to fipronil (2.8×). The Boat Quay and Ghimmoh Road strains showed low resistance to fipronil, whereas the remaining strains (Beach Road, Boat Quay, and Victoria Street strains) were susceptible to fipronil.

Fipronil Synergism. Neither PBO nor DEF had a synergistic effect on fipronil toxicity in any of the six strains (Table 2). However, PBO had a significant antagonistic effect on fipronil toxicity in all strains of cockroach. Fipronil toxicity decreased after the test

Table 1. Toxicity of fipronil and dieldrin against six strains of the German cockroach at 48 h posttreatment

Insecticide	Strain	*n*	LD_{50} (95% FL) (μg/g)	LD_{95} (95% FL) (μg/g)	Slope	χ^2 (df)	RR_{50}[a]
Dieldrin	EHI (susceptible)	120	4.90 (4.47–5.34)	7.75 (6.88–9.37)	8.26 ± 1.17	0.05 (1)	—
	B1 Tampines Central	160	6.35 (4.35–7.91)	27.80 (20.04–54.39)	2.57 ± 0.50	1.02 (2)	1.3
	Beach Road	160	5.22 (2.65–8.52)	15.69 (9.32–101.65)	3.44 ± 0.44	2.72 (2)	1.1
	Boat Quay	160	6.36 (3.56–9.70)	18.93 (11.67–100.86)	3.47 ± 0.46	2.38 (2)	1.3
	Victoria Street	200	8.93 (4.57–20.10)	75.76 (28.10–8749.96)	1.77 ± 0.28	0.37 (3)	1.8
	Cavenagh Road	350	20.09 (11.25–78.52)	292.90 (76.23–98806.00)	1.41 ± 0.19	17.60 (5)	4.1*
	Ghimmoh Road	150	6.94 (3.47–11.08)	29.31 (15.93–358.77)	2.63 ± 0.41	5.10 (3)	1.4
Fipronil	EHI (susceptible)	120	0.05 (0.05–0.06)	0.10 (0.08–0.13)	6.41 ± 0.96	1.97 (2)	—
	B1 Tampines Central	210	0.08 (0.08–0.08)	0.11 (0.10–0.12)	12.94 ± 1.98	0.60 (4)	1.6*
	Beach Road	200	0.06 (0.05–0.07)	0.11 (0.09–0.20)	6.16 ± 0.82	3.48 (3)	1.2
	Boat Quay	180	0.07 (0.05–0.09)	0.12 (0.09–0.31)	6.41 ± 0.78	13.24 (4)	1.4
	Victoria Street	150	0.06 (0.04–0.09)	0.14 (0.10–0.80)	4.59 ± 0.59	8.52 (3)	1.2
	Cavenagh Road	200	0.15 (0.09–0.27)	0.48 (0.26–7.62)	3.22 ± 0.40	9.18 (3)	3.0*
	Ghimmoh Road	120	0.11 (0.09–0.13)	0.29 (0.22–0.47)	3.84 ± 0.63	0.81 (2)	2.2*

[a] Asterisk denotes that the toxicity was significantly different from that of the susceptible strain based on nonoverlap of the 95% FL.

insects were pretreated with PBO. A similar observation was made when DEF, an esterase inhibitor, was used as the synergist, as DEF also decreased fipronil toxicity. The RRs of all resistant strains pretreated with synergists (0.9–2.5×) were slightly reduced from the values when insecticide alone was used (1.2–3.0×) (Table 2). These results suggest that P_{450} cytochrome and esterase were likely not the mechanisms contributing to fipronil resistance in the strains of German cockroach used in this study.

Bait Evaluation. All field-collected strains of cockroach were susceptible to the fipronil bait (Table 3). The LT_{50} of the EHI-susceptible strain was 8.26 h (7.72–8.84). The LT_{50} values for most of the field-collected strains were in the range of 7–10 h, but there were no significant differences between resistant and EHI-susceptible strains based on nonoverlap of the 95% FLs (with the exception of the Cavenagh Road strain). The Cavenagh Road strain exhibited a low tolerance to fipronil gel bait, with an RR of 1.1×.

Bait Selection. Selection with fipronil bait via topical application significantly increased the LD_{50} (95% FL) of fipronil from 0.07, 0.15, and 0.11 μg/g in the parental generation of the Boat Quay, Cavenagh Road, and Ghimmoh Road strains, respectively, to 0.66, 1.55, and 0.65 μg/g in the F_5 generation, respectively (Table 4). Dieldrin resistance in the selected cockroach populations was significantly increased up to 4,000 fold in the Cavenagh Road F_5 strain (Table 4). A significant increase in the RR was also found in the F_5 generation of the three fipronil-selected strains of cockroach compared with the parental generation (Fig. 2). Table 5 lists the evaluation of effec-

Table 2. Synergism of fipronil + PBO and/or DEF against six strains and three selected populations of the German cockroach

Strain	Synergists	Generation	*n*	LD_{50} (95% FL) (μg/g)	LD_{95} (95% FL) (μg/g)	Slope	χ^2 (df)	SR_{50}	RR_{50}[a]
EHI (susceptible)	PBO	—	120	0.07 (0.05–0.09)	0.34 (0.18–6.49)	2.37 ± 0.80	0.42 (2)	0.7	—
	DEF	—	90	0.12 (0.10–0.18)	0.27 (0.18–0.83)	4.71 ± 1.18	0.30 (1)	0.4	—
	PBO+DEF	—	90	0.09 (0.08–0.10)	0.16 (0.13–0.27)	6.65 ± 1.61	0.40 (1)	0.6	—
B1 Tampines	PBO	—	150	0.09 (0.08–0.10)	0.18 (0.15–0.24)	5.76 ± 1.04	0.20 (3)	0.9	1.3
Central	DEF	—	120	0.14 (0.12–0.16)	0.29 (0.23–0.45)	5.07 ± 0.92	1.97 (2)	0.4	1.2
	PBO+DEF	—	120	0.13 (0.12–0.15)	0.27 (0.22–0.39)	5.31 ± 0.93	1.56 (2)	0.3	1.4*
Beach Road	PBO	—	150	0.12 (0.09–0.15)	0.20 (0.15–0.43)	7.53 ± 1.00	5.94 (3)	0.5	1.3
	DEF	—	120	0.16 (0.14–0.18)	0.36 (0.27–0.67)	4.53 ± 0.94	0.50 (2)	0.2	1.3
	PBO+DEF	—	90	0.11 (0.10–0.12)	0.18 (0.16–0.26)	7.72 ± 1.82	0.32 (1)	0.3	1.2
Boat Quay	PBO	P	180	0.12 (0.11–0.13)	0.20 (0.18–0.24)	7.13 ± 0.87	2.43 (4)	0.6	1.7*
		F_5	150	0.67 (0.44–0.85)	3.01 (2.17–5.79)	2.51 ± 0.49	3.81 (3)	0.8	9.6*
	DEF	P	120	0.15 (0.13–0.16)	0.30 (0.24–0.45)	5.28 ± 0.94	0.84 (2)	0.2	1.3
	PBO+DEF	P	120	0.08 (0.07–0.10)	0.26 (0.20–0.41)	3.42 ± 0.53	1.96 (2)	0.3	0.9
Victoria Street	PBO	—	200	0.11 (0.09–0.12)	0.29 (0.23–0.41)	3.78 ± 0.49	1.78 (3)	0.5	1.6
	DEF	—	120	0.17 (0.15–0.20)	0.36 (0.28–0.58)	5.19 ± 0.98	0.53 (2)	0.2	1.4
	PBO+DEF	—	90	0.14 (0.12–0.15)	0.22 (0.19–0.28)	8.57 ± 1.98	0.01 (1)	0.3	1.6*
Cavenagh Road	PBO	P	200	0.15 (0.13–0.19)	0.30 (0.24–0.47)	5.63 ± 0.58	3.21 (3)	1.0	2.1*
		F_5	120	0.96 (0.64–1.26)	6.47 (3.39–48.97)	1.98 ± 0.54	2.61 (2)	1.3	13.7*
	DEF	P	120	0.24 (0.20–0.38)	0.75 (0.45–3.14)	3.36 ± 0.82	0.15 (2)	0.2	2.0*
	PBO+DEF	P	120	0.14 (0.09–0.20)	1.05 (0.48–40.67)	1.89 ± 0.63	0.14 (2)	0.1	1.6
Ghimmoh Road	PBO	P	150	0.16 (0.11–0.20)	0.28 (0.22–1.04)	6.26 ± 0.94	6.42 (3)	0.7	2.3*
		F_5	150	0.70 (0.44–0.90)	3.39 (2.39–6.92)	2.40 ± 0.48	3.60 (3)	0.8	10.0*
	DEF	P	120	0.18 (0.16–0.21)	0.36 (0.29–0.59)	5.52 ± 1.05	1.14 (2)	0.3	1.5
	PBO+DEF	P	90	0.12 (0.09–0.14)	0.27 (0.20–1.37)	4.71 ± 1.68	0.54 (1)	0.4	1.3

[a] Resistance ratio (RR_{50}) was determined by dividing the LD_{50} of resistant strains (+synergist) by the corresponding value of the EHI-susceptible strain (+synergist).

Table 3. Evaluation of effectiveness of Goliath (fipronil 0.05%) cockroach gel bait against six strains of German cockroach

Strain	*n*	LT_{50} (95% FL) (h)	LT_{95} (95% FL) (h)	Slope	χ^2 (df)	RR_{50}[a]
EHI (susceptible)	40	8.26 (7.72–8.84)	17.92 (15.53–22.01)	4.89 ± 0.49	2.28 (8)	—
B1 Tampines Central	40	7.50 (7.05–7.97)	14.91 (13.27–17.52)	5.51 ± 0.50	2.31 (8)	0.9
Beach Road	40	7.74 (6.98–8.73)	26.83 (20.34–41.32)	3.05 ± 0.36	1.49 (8)	0.9
Boat Quay	40	7.50 (6.97–8.06)	16.77 (14.51–20.70)	4.71 ± 0.49	1.61 (7)	0.9
Victoria Street	40	8.57 (7.34–10.75)	36.82 (23.54–85.76)	2.60 ± 0.42	1.21 (7)	1.0
Cavenagh Road	40	9.45 (8.87–10.07)	20.29 (17.51–25.31)	4.95 ± 0.54	4.12 (8)	1.1*
Ghimmoh Road	40	6.97 (6.49–7.44)	14.87 (13.21–17.56)	5.00 ± 0.48	3.90 (8)	0.8

[a] Asterisk denotes that the toxicity was significantly different from that of the susceptible strain based on nonoverlap of the 95% FL

tiveness of fipronil cockroach gel bait against all generations of the three selected cockroach strains. There were significant increases in LT_{95} between F_1 and F_5 generations in all three selected cockroach strains.

Relationship Between Fipronil and Dieldrin Susceptibility. Susceptibility of the cockroach strains to fipronil correlated positively with susceptibility to dieldrin (Fig. 3). The relationship between the toxicity of dieldrin and fipronil was determined by using the results obtained from the parental, F_3, and F_5 generations of the three fipronil-selected cockroach strains. Correlation analysis revealed a significant positive correlation between the LD_{50}s of dieldrin and those of fipronil ($P < 0.01$, $R = 0.83$). Further analysis revealed a linear regression model, $y = (6952.69 \pm 1988.37)x - (708.93 \pm 1226.28)$, ($F = 12.22$; $P < 0.05$; $R^2 = 0.43$), where $y = LD_{50}$ of dieldrin, $x = LD_{50}$ of fipronil, and $x > 0$ (Fig. 3).

Detection of the *Rdl* Mutation. A single fragment of the *Rdl* gene (245 bp) was amplified and sequenced from the EHI-susceptible and all three fipronil-selected strains by using forward and reverse primers (Hansen et al. 2005, Gondhalekar and Scharf 2012). Based on previous studies (Gondhalekar and Scharf 2012), there are three genotypes (Ser/Ser, Ser/Ala,

Table 4. Toxicity of dieldrin and fipronil against three fipronil-selected strains and the EHI-susceptible strain of German cockroaches

Insecticide	Strain	Generation	*n*	LD_{50} (95% FL) (μg/g)	Slope	χ^2 (df)	RR_{50}[a]
Dieldrin	EHI (susceptible)	—	90	4.74 (4.38–5.20)	8.52 ± 1.76	0.48 (1)	—
		—	90	4.81 (4.46–5.28)	8.83 ± 1.78	1.89 (1)	—
		—	120	4.73 (4.49–4.98)	13.45 ± 2.01	1.24 (1)	—
		—	90	4.50 (3.98–5.09)	5.99 ± 1.58	0.58 (1)	—
		—	120	4.55 (4.02–5.18)	4.96 ± 1.33	0.88 (1)	—
	Boat Quay	F_2	120	17.82 (13.24–36.73)	1.83 ± 0.58	0.61 (2)	3.8*
		F_3	240	18.09 (10.19–25.40)	1.73 ± 0.28	4.36 (4)	3.8*
		F_4	160	3,177.54 (2,005.94–4,773.72)	1.25 ± 0.33	1.98 (2)	671.8*
		F_5	140	6,851.36 (5,427.94–8,192.42)	2.65 ± 0.59	3.28 (2)	1,522.5*
	Cavenagh Road	F_2	150	52.45 (32.61–239.62)	1.11 ± 0.36	1.50 (3)	11.1*
		F_3	180	117.20 (88.70–194.34)	1.71 ± 0.38	1.20 (4)	24.4*
		F_4	150	6,052.78 (4,154.75–10,831.23)	1.76 ± 0.28	0.82 (4)	1,279.7*
		F_5	160	18,090.33 (16,357.40–21,277.01)	7.12 ± 1.35	0.21 (2)	4,020.1*
	Ghimmoh Road	F_2	120	26.83 (18.83–65.69)	1.51 ± 0.54	0.64 (2)	5.7*
		F_3	180	36.54 (26.40–54.17)	1.39 ± 0.30	0.45 (4)	7.6*
		F_4	180	147.97 (110.80–213.09)	1.55 ± 0.36	1.82 (4)	31.3*
		F_5	120	7,453.15 (6,080.50–9,354.87)	2.87 ± 0.69	0.13 (2)	1,656.3*
Fipronil	EHI (susceptible)	—	160	0.11 (0.10–0.12)	4.02 ± 0.82	2.72 (2)	—
		—	120	0.09 (0.07–0.11)	3.90 ± 0.86	1.88 (2)	—
		—	120	0.07 (0.06–0.09)	2.83 ± 0.62	0.44 (2)	—
		—	150	0.07 (0.04–0.13)	4.26 ± 0.64	8.77 (3)	—
		—	120	0.06 (0.03–0.08)	5.77 ± 0.81	7.78 (3)	—
		—	120	0.08 (0.07–0.08)	12.71 ± 2.08	0.30 (2)	—
	Boat Quay	F_1	160	0.11 (0.10–0.12)	6.28 ± 0.87	1.26 (2)	1.4
		F_2	150	0.14 (0.12–0.17)	3.23 ± 0.56	1.55 (3)	1.5*
		F_3	150	0.15 (0.13–0.17)	3.49 ± 0.68	0.36 (3)	2.1*
		F_4	140	0.54 (0.46–0.62)	4.91 ± 0.76	0.47 (2)	7.7*
		F_5	120	0.66 (0.55–0.79)	3.44 ± 0.54	0.79 (2)	11.0*
	Cavenagh Road	F_1	240	0.20 (0.17–0.23)	3.41 ± 0.47	3.33 (4)	2.5*
		F_2	120	0.43 (0.29–0.55)	2.23 ± 0.44	1.48 (2)	4.8*
		F_3	180	1.04 (0.70–2.08)	1.33 ± 0.30	0.36 (3)	14.9*
		F_4	120	1.25 (0.29–2.70)	4.48 ± 0.65	4.06 (2)	17.9*
		F_5	160	1.55 (1.36–1.73)	4.42 ± 0.69	0.80 (2)	25.8*
	Ghimmoh Road	F_1	200	0.14 (0.12–0.17)	5.50 ± 0.74	3.33 (3)	1.8*
		F_2	90	0.19 (0.16–0.22)	5.21 ± 1.17	0.97 (1)	2.1*
		F_3	120	0.23 (0.20–0.30)	3.70 ± 0.79	1.47 (2)	3.3*
		F_4	140	0.53 (0.41–0.64)	2.80 ± 0.45	1.93 (2)	7.6*
		F_5	150	0.65 (0.17–1.29)	2.78 ± 0.42	8.90 (3)	10.8*

[a] Asterisk denotes that the toxicity was significantly different from that of the susceptible strain based on nonoverlap of the 95% FL.

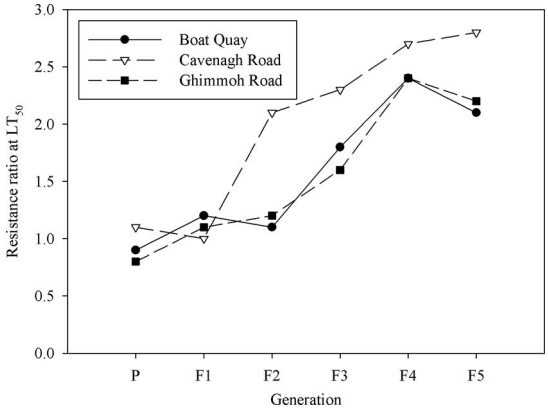

Fig. 2. Resistance ratio at LT_{50} of several generations of three fipronil-selected cockroach strains determined by bait assays from the parental to the F_5 generation.

and Ala/Ala) at position 302 in the *Rdl* gene of *B. germanica.* As expected, all tested individuals from the EHI-susceptible strain were susceptible homozygotes (Ala/Ala) (Table 6). Comparison of the *Rdl* DNA sequence of the three fipronil-selected populations (parental and F_5 generation) with that of the EHI-susceptible strain revealed the existence of an A302S mutation in all three selected populations. Seven of 20, 6 of 20, and 3 of 20 individuals from the Boat Quay, Cavenagh Road, and Ghimmoh Road strains, respectively, were genotyped as heterozygous (Ala/Ser) for the *Rdl* mutation before the laboratory selection process. Only 20 and 5% of individuals from the Cavenagh Road and Ghimmoh Road strains, respectively, were homozygous resistant (Ser/Ser). The remaining individuals were homozygous susceptible (Ala/Ala). After five generations of bait laboratory selection, we found all individuals from the two fipronil-selected strains, Cavenagh Road-F_5 and Boat Quay-F_5, to be homozygously resistant (Ser/Ser). However, we detected only 85% of the tested individuals of Ghimmoh Road strain were homozygous resistant, whereas the remaining were heterozygous individuals (Table 6). The amino acid substitution from Ala to Ser resulted in a single nucleotide polymorphism from guanine to thymine. Detection of a high frequency of the homozygous resistant allele, Ser/Ser, in all selected cockroach populations suggests that the A302S mutation in the *Rdl* gene is associated with fipronil and dieldrin resistance in our cockroach populations.

Discussion

Low fipronil resistance was detected in several field-collected strains of German cockroach through topical bioassay. Based on the treatment history, these populations had no previous exposure to fipronil before they were collected. Holbrook et al. (2003) reported a similar situation in which field cockroaches that had no pre-exposure history to this compound were resistant to topically administered and ingested fipronil. One of the field-collected populations in the current study, Cavenagh Road, exhibited low resistance to both fipronil and dieldrin. This finding suggests that past exposure to dieldrin may have selected for individuals that carried the *Rdl* gene and that although dieldrin had not been used for a long time, the *Rdl* gene continued to be maintained in the population without any fitness loss. This prediction was confirmed by detection of a low level of the *Rdl* mutation in the parental generation of the three cockroach strains before the laboratory selection process in the current study. Previously, Ang and Lee (2011) reported absence of reduced fitness in the same field-collected cockroach strains. This premise was also strongly supported by Holbrook et al. (2003), who suggested that fipronil resistance had been only min-

Table 5. Evaluation of effectiveness of Goliath (fipronil 0.05%) cockroach gel bait against three selected cockroach strains for every generation

Strain	Generation	*n*	LT_{95} (95% FL) (h)	Slope	χ^2 (df)	RR_{95}[a]
EHI (susceptible)	—	40	15.43 (14.11–17.34)	5.97 ± 0.47	4.03 (10)	—
	—	40	15.65 (14.04–18.19)	5.88 ± 0.54	3.48 (8)	—
	—	40	8.29 (7.58–9.46)	8.00 ± 0.94	3.60 (4)	—
	—	40	8.76 (7.72–10.91)	5.95 ± 0.95	1.41 (4)	—
	—	40	21.84 (19.92–24.73)	6.00 ± 0.52	6.59 (12)	—
Boat Quay	F_1	40	25.77 (21.15–33.54)	4.03 ± 0.43	2.55 (6)	1.7*
	F_2	40	17.35 (15.56–20.13)	5.68 ± 0.49	3.22 (9)	1.1
	F_3	40	17.80 (15.83–20.95)	5.80 ± 0.54	3.99 (7)	2.1*
	F_4	40	21.01 (18.33–25.64)	6.09 ± 0.62	5.45 (8)	2.4*
	F_5	40	36.40 (33.96–40.33)	9.65 ± 1.14	0.55 (8)	1.7*
Cavenagh Road	F_1	40	20.10 (17.32–24.81)	4.34 ± 0.41	3.35 (9)	1.3
	F_2	40	51.03 (40.50–71.70)	3.52 ± 0.36	4.52 (8)	3.3*
	F_3	40	26.59 (23.26–31.78)	4.61 ± 0.38	4.10 (10)	3.2*
	F_4	40	29.85 (24.55–39.92)	4.38 ± 0.47	3.63 (7)	3.4*
	F_5	40	51.87 (47.05–60.05)	7.86 ± 0.85	1.73 (9)	2.4*
Ghimmoh Road	F_1	40	17.82 (15.65–21.43)	5.20 ± 0.50	1.12 (8)	1.2
	F_2	40	25.71 (21.97–32.10)	4.03 ± 0.38	2.65 (10)	1.6*
	F_3	40	15.10 (13.64–17.39)	6.24 ± 0.59	3.72 (7)	1.8*
	F_4	40	19.55 (17.10–24.16)	6.54 ± 0.80	1.14 (6)	2.2*
	F_5	40	54.47 (48.96–62.56)	4.94 ± 0.37	1.16 (15)	2.5*

[a] Asterisk denotes that the toxicity was significantly different from that of the susceptible strain based on nonoverlap of the 95% FL.

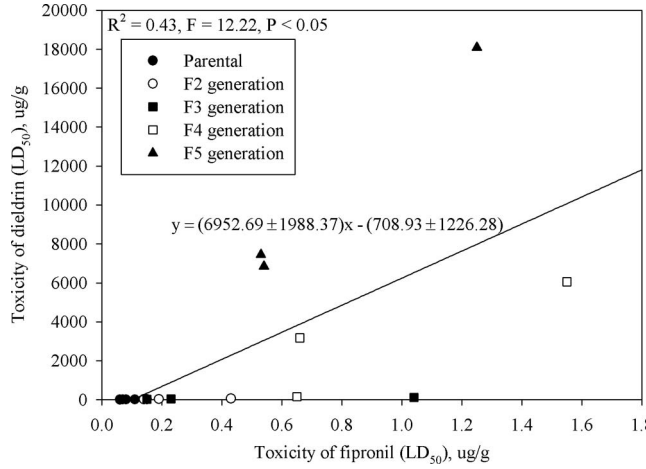

Fig. 3. Linear relationship between LD_{50} of dieldrin and LD_{50} of fipronil in field-collected strains of German cockroach.

imally diminished because of limited differences in the fitness of resistant and susceptible individuals. This result is consistent with several studies in other pest insects in which absence of fitness cost was detected in laboratory and field insect populations carrying the *Rdl* mutation (Aronstein et al. 1995, Bass et al. 2004).

P_{450} monooxygenase was reported to be involved in fipronil resistance in *Musca domestica* (L.) (Wen and Scott 1999, Liu and Yue 2000), *Chilo suppressalis* (Walker) (Li et al. 2007), *Spodoptera litura* (F.) (Ahmad et al. 2008), and *Cimex hemipterus* (F.) (How and Lee 2011), but it seemed to be antagonistic to fipronil toxicity in the western corn rootworm *Diabrotica virgifera* (LeConte) (Scharf and Siegfried 1999), European corn borer *Ostrinia nubilalis* (Hübner) (Durham et al. 2001), and *B. germanica* (Scott and Wen 1997, Valles et al. 1997). In contrast, a recent study (Gondhalekar and Scharf 2012) documented the involvement of cytochrome P_{450} in fipronil resistance in a field-collected population of the German cockroach through a synergism bioassay. The authors (Gondhalekar and Scharf 2012) found that reduced penetration of the insecticide was not a resistance mechanism and that preapplication of a synergist did not affect fipronil penetration through the cockroach cuticle. Thus, the effect of the monooxygenase enzyme was believed to be strain dependent instead of species dependent. Our result was in agreement with previous studies (Scott and Wen 1997, Valles et al. 1997) showing that fipronil became less toxic when the cockroaches were pretreated with the synergists PBO (a monooxygenase inhibitor) or DEF (an esterase inhibitor) compared with fipronil alone. These results were further supported by Mulrooney and Goli (1999), Durham et al. (2002), and Zhao et al. (2005), who reported that bioactivation of fipronil to form the more toxic sulfone metabolite is catalyzed by cytochrome P_{450}-mediated microsomal monooxygenase in insects. Fang et al. (2008) suggested that higher activity of mixed function oxidases in *C. suppressalis* can produce higher amount of fipronil-sulfone. Surprisingly, DEF, an esterase inhibitor, also antagonized fipronil toxicity, and it was more effective than PBO among our cockroach strains. DEF is not a completely specific esterase inhibitor, and it may result in monooxygenase inhibition to a certain degree (Scott 1990). Biochemical assays of monooxygenase, general esterase, and glutathione-S-transferase on F_3 generation of similar cockroach strains revealed no significant correlation between fipronil toxicity and any of the enzyme activities measured. Thus, enhanced metabolic detoxification may not be the main mechanism that confers fipronil resistance in these strains.

There was a positive relationship between the LD_{50} values of dieldrin and those of fipronil in the strains tested, suggesting cross-resistance between these two

Table 6. Distribution of *Rdl* genotypes in the EHI-susceptible and three fipronil-selected (parental and F_5 generation) strains of the German cockroach

Strain	Generation	*n*	No. genotypes		
			Ala302/Ala302 (S/S)	Ala302/Ser302 (R/S)	Ser302/Ser302 (R/R)
EHI (susceptible)	—	20	20	0	0
Boat Quay	P	20	13	7	0
	F_5	20	0	0	20
Cavenagh Road	P	20	10	6	4
	F_5	20	0	0	20
Ghimmoh Road	P	20	16	3	1
	F_5	20	0	3	17

insecticides. Several studies reported relatively low cross-resistance between fipronil and cyclodiene in some pest insects, including *M. domestica* (Scott and Wen 1997, Kristensen et al. 2004), *Anopheles stephensi* (Liston) (Davari et al. 2007), and *B. germanica* (Scott and Wen 1997, Holbrook et al. 2003, Kristensen et al. 2005). Selection with dieldrin caused positive cross-resistance with fipronil in the mosquito *An. stephensi*, and it was predicted that GABA channel mutation might be involved (Davari et al. 2007). Scott and Wen (1997) reported that three dieldrin-resistant strains of German cockroach and house fly were cross-resistant to fipronil, with RRs of 6.7–7.7, 15, and 31-fold, respectively. Holbrook et al. (2003) also detected a strong positive linear relationship between mortality of males that were treated with fipronil and mortality rate when treated with dieldrin. Given that both insecticides share a similar target site (i.e., the GABA receptor [Cole et al. 1993, Hosie et al. 1995, Gant et al. 1998, Narahashi 2002, Buckingham and Sattelle 2005]), cross-resistance is expected.

Recently, Gondhalekar and Scharf (2012) identified A302S-encoding mutation in the *Rdl* gene in a highly fipronil-resistant field-collected cockroach strain, GNV-R. Hansen et al. (2005) found a similar mutation in German cockroaches that confers high dieldrin resistance in homozygous individuals and moderate resistance in heterozygotes in the Danish populations of *B. germanica.* Kristensen et al. (2005) documented a causal connection between the frequency of *Rdl* mutation and dieldrin and fipronil resistance. Furthermore, Gondhalekar and Scharf (2012) used logistic regression analysis of electrophysiology data to show that A302S mutation frequency was correlated with neurological insensitivity. *Rdl* mutation was also reported to potentially alter the rate of desensitization, stabilize the open conformation of GABA receptor, and reduce the potency of fipronil (Zhang et al. 1994). Thus, these studies verified the effect of *Rdl* mutation in fipronil resistance in the German cockroach. Hosie et al. (1995) revealed that fipronil at 1–100 μM blocked the dieldrin-sensitive GABA receptor at a higher rate compared with the dieldrin-resistant (*Rdl* mutation) GABA receptor. The differential inhibition rate between these two types of receptor is evidence that the *Rdl* mutation likely interacts physically with the fipronil compounds or may indirectly alter the allosteric linking mechanism on the fipronil binding site (Zhang et al. 1994). Based on the aforementioned studies, we conclude that the *Rdl* mutation is the mechanism that confers cross-resistance between dieldrin and fipronil, despite their different binding effects on the targeted channel. The current study documented that mostly resistant homozygous individuals were detected in the 302 *Rdl* gene in all F_5 generation of fipronil-selected populations of German cockroach. Although several individuals from Ghimmoh Road were heterozygotes, the results strongly support the premise that A302S mutation was involved in fipronil and dieldrin resistance, with RRs of 10.8–25.8× and 1500–4000×, respectively, in the strains that were subjected to five generations of laboratory bait selection. Although the frequency of the resistant allele was unexpectedly high (92.3–100%), fipronil resistance in our fipronil-selected cockroach populations remained at a moderate level. A similar situation was reported in one of the Danish cockroach populations by Kristensen et al. (2005) and Hansen et al. (2005), who documented moderate and high level of fipronil (15×) and dieldrin resistance (1,270×), respectively, in the Zo960302 strain at the resistant allele frequency of 97%. In addition, the authors (Hansen et al. 2005, Kristensen et al. 2005) also discovered 37 of 38 individuals were resistant homozygotes in a similar strain. The resistant homozygotes for A302S mutation have also been documented in natural populations of other insect species, such as cat flea, *Ctenocephalides felis* (Bouché) (Bass et al. 2004, Brunet et al. 2009), and diamondback moth, *Plutella xylostella* (L.) (Li et al. 2006). Thus, we strongly believe that there will be no potential tradeoffs for *Rdl*-resistant homozygotes.

The differences between the magnitude of dieldrin resistance and that of fipronil detected in the current study may suggest differences in their mode of action. The results were consistent with some previous studies in which the level of fipronil resistance was ≈100, 150, and 2,000-fold lower than the level of dieldrin resistance in cockroach strains Zo960302, Ga021001 (Kristensen et al. 2005), and Cld-R (Scott and Wen 1997), respectively. This difference was because of their differential biochemical actions on cockroach neurons (Raymond et al. 2000, Narahashi 2002, Zhao et al. 2003). Fipronil exerted only one potent partially reversible inhibitory action on the GABA receptor, whereas dieldrin exhibited an irreversible dual action (potentiating action followed by inhibition). In addition, the effect of fipronil was 10-fold faster than that of dieldrin on the cockroach GABA receptor (Zhao et al. 2003), and it is also readily metabolized via oxidation to form a byproduct (fipronil-sulfone) that is more toxic than the parental compound (Valles et al. 1997, Hainzl et al. 1998, Zhao et al. 2005, Zhao and Salgado 2010). Both parental and bioactivated compounds were reported to exhibit a neurophysiological effect on the GluCI receptor (Zhao et al. 2004). Although the effect of A302S *Rdl* mutation on the sensitivity of GluCI subunits to fipronil remains unclear, the effect of fipronil on GluCIs could be one of the important factors limiting fipronil resistance (Nakao et al. 2011).

Rdl mutation in the German cockroach is common and widely distributed throughout the world. The first case *Rdl* mutation was reported by Kristensen et al. (2005) in a Danish cockroach population, and Gondhalekar and Scharf (2012) later found it in a U.S. cockroach population. In this study, we reported the first case of *Rdl* mutation in the German cockroach from Asia. The use of cyclodienes in cockroach control in the late 1960s and early 1970s and the lack of fitness disadvantages of the *Rdl* resistance allele during absence of selection pressure in cockroach populations likely have contributed to this prefipronil resistance situation. To date, only a single mutation (A302S) on the GABA receptor has been detected in the German

cockroach. Although the fipronil resistance observed in three strains studied herein was artificially created, it strongly suggests that the resistant allele existed in these cockroach populations at the point of collection and was maintained in the laboratory without any fitness loss. The prevalence of the *Rdl* mutation in field populations of the German cockroach in Asia remains unknown at this time. More studies are required to further substantiate this issue.

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